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Studies of Endophytic Actinomycetes Associated with Medicinal Plants of Mizoram, Northeast, India

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ABSTRACT

Keywords

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A total of 17 endophytic actinomycete strains were obtained from 6 medicinal plants of Mizoram, Northeast, India. 16S rRNA results showed 13 (73.3%) of the isolates belonged to the genus *Streptomyces*, 2 (13.3%) were *Nocardiopsis*, 1 (6.6%) were *Tsukamurella tyrosinosolvens* and 1 (6.6%) *Actinobacteria bacterium*. The highest number of endophytic actinomycetes was isolated from root tissue of *Mikania micrantha* Kunth. WI. (29.4%) followed by stem of *Mikania micrantha* Kunth. WI. and rhizome of *Costus speciosus* (J. Konig). Eight endophytic Streptomyces showed activity against two major phytopathogenic fungi: *Fusarium oxysporum f. sp. ciceri* (MTCC-2791) and *Fusarium proliferatum* (*MTCC-286*). Six isolates showed solubilisation of inorganic phosphorous. All the isolates showed abilities to produce indole-3-acetic acid (IAA) and ammonia. These results clearly suggested that endophytic actinomycetes were alternative source as bioinoculents for plant growth promotion or as well as biocontrol agent for sustainable agricultural developments.

Introduction

Endophytes means microorganisms live inside the tissues of living plants without any effect of infection of the host plants (Strobel et al., 2004). Endophytic microorganisms are well known for production of diverse range of secondary metabolites (Strobel and Daisy, 2003; Fiedler et al., 2008; Schulz et al., 2009). The mutual relationship of microorganisms with plants found various benefits to the host plants such as production of antimicrobials, protection against plant environmental plant growth promoting stresses. phytohormones (Bailey et al., 2006; Clegg and Murray, 2000). Actinomycetes are Grampositive bacteria, saprophytic soil inhabitants, ubiquitous in nature. Actinomycetes found as one of the important group of organisms from various plant tissues of the world (Sardi et al., 1992; De-Araujo et al., 2000; Cao et al., 2004; Coombs and Franco, 2003) including various Asteraceae family (Tanvir et al., 2014) for their producing potential bioactive compounds and various novel compounds (Igarashi et al., 2007). Among the 10,000 antimicrobial compounds produced by microorganisms, more than 50 % were isolated from actinomycetes and about 60% of the bioactive compounds developed for agricultural use were originated from genus Streptomyces (Anderson and Wellington, 2001). Medicinal

plants have been important foundations for large number of secondary metabolites which is highly used in pharmaceutical industries, food industries and further biomedical studies. According to the previous researchers, potential and high diversity of actinomycetes were examined from several medicinal plants (Khamna et al., 2009; Qin et al., 2011; Zhao et al., 2011; Taechowisan and Lumyong, 2003; Verma et al., 2009; Passari et al., 2015). Strobel and Daisy, 2003 suggested that plant selection is very tactical, plants with rare location and biology with traditional ethnobotanical history should be chosen for isolating endophytes producing novel bioactive products.

The diversity of endophytic actinomycetes may changes between different plant species and regions (Qin et al., 2011). Northeastern (NE) Region of India is a big bioprospecting area and best known for its rich biodiversity and un-tapped bioresources which has been identified as a significant position of both the Himalaya and Indo-Burma biodiversity hotspots (Myers et al., 2000). Mizoram is an important States of Northeastern India and also is a part of the 25 mega-biodiversity hotspots of the world. However, documented medicinal plants with ethnobatnical history has been reported more species by past researchers (Lalramnghinglova and Jha, 1998; Rai and Lalramnghinglova, 2010). There are very few reports on the studies of endophytic actinomycetes residing in the traditional medicinal plants of these regions. Therefore, endophytic actinomycetes in these regions remain unexplored and uncharacterized. This importance encouraged us to close examinations this habitat to understand the role of endophytic actinomycetes from medicinal plants of Mizoram, Northeast, India.

The present study deals with the isolation and identification of endophytic actinomycetes

associated with ethno-medicinal plants of Mizoram, Northeast, India. The isolates were characterized relating with plant growth promoting activities.

Materials and Methods

Collection of plant samples

Six different medicinal plants viz., Senecio scandens Buch.-Ham. Mikania micrantha Kunth. WI, Ageratum conizoides L., Costus speciosus (J. Konig) Sm., Cassia fistula L., Scopariadulcis (Table 1) were collected from two districts, Aizawl district (23° 45'N; 92° 38 'E) collection site located about 15-20km from the main city and Mamit district (23°25 'N; 92° 20' E) of Mizoram, Northeast, India, 122.7km distance away from the capital city Aizawl. Plants were selected based on their traditional used of medicinal plants and their abundance. Plants tissues (root, stem, petiole, flower, rhizome) were taken carefully in a sterile polythene bags and brought to the laboratory of Biotechnology, Mizoram University, Mizoram. Tissues were kept at 4°C processed isolation of endophytic actinomycetes within 24-48hours collection.

Isolation of endophytic actinomycetes

All the collected samples were washed thoroughly in running tap water to remove dust particles and organic debris. Tissues from leaves, stem, flower, root were cut into 1cm² small pieces and perform surface-sterilization. The sterile tissues were placed onto prepared medias: Starch Casein Agar (SCA), Starch Casein Nitrate Agar (SCNA), Tryptone Soya Agar (TSA), Glycerol Aspargine Agar (ISP₅) supplemented with antibiotics: Nalidixic acid to inhibit the negative gram of bacterial growth, Nystatin and Cycloheximide to suppress the fungal growth according to methods given by

Morphological and microscopically identification of isolated endophytic actinomycetes

Dry, fuzzy, sticky, hard, filamentous colonies were observed as actinomycetes and were subculture by streaking method repeatedly till pure culture obtained (Fig. 1). Purified isolates were identified according to Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994). Microscopically observation for their chain morphology under Field Emission Gun-Scanning Electron Microscopy (FEG-SEM) (Kumar *et al.*, 2011) (Fig. 2).

Molecular identification of isolated endophytic actinomycetes

Extraction of genomic DNA was carried out by using the Pure Link Genomic DNA Extraction kit (IN-vitrogen, Carllbad, CA USAD). The 16S rRNA gene was amplified as described by Cui et al., 2001, using universal primers set PA (5'-AGAGTTTGATCCTGGCTCA-3') and PH (5'-ACGGCTACCTTGTTACGACT-3'). The DNA sequencing was sent commercially (Sci-Genome Labs Private Ltd., Chennai, India). The sequence nucleotides of the isolates were determined through Blast search using the NCBI databases and deposited in NCBI Gen Bank.

In vitro antagonistic activity of endophytic actinomycetes

All isolates were evaluated for antagonistic activity against major fungal two phytopathogens, Fusarium oxysporum f. sp. ciceri (MTCC-2791) Fusarium and proliferatum (MTCC-286) by dual culture in vitro assay (Bredholdt et al., 2007). The pathogens were obtained from Microbial Type Culture Collection, Institute of Microbial Technology (IMTECH), Chandigarh, India. Plates with only pathogen culture were served as control. All plates were incubated at 28 0C

and percentage of inhibition was calculated by using the formula C-T/C x 100, where, C is the colony growth of fungal pathogen in control, and T is the colony growth in dual culture.

Phosphate solubilisation

Qualitative phosphate solubilisation of isolates were analysed on Pikovskaya's agar media, considered to have P-solubilization to those forming clear halo zone around their colonies (Nautiyal *et al.*, 1999).

Indole acetic acid (IAA) production

The culture was grown on ISP₂ media incubated at 28°C. Four-millimetre-diameter agar disc were cut using sterile borer and inoculated into 100ml of ISP₂ broth containing 0.2% L-Tryptophan. The culture was grown with continuous shaker at 125rpm. After incubation the suspension was centrifuged and 1ml supernatant was mixed with 2ml of Salkowski's reagent and further incubated in dark for 30 minutes. IAA was observed as the development of a pink-red colour (Gordon and weber, 1951).

Ammonia production

The culture was inoculated at 10ml of 4% peptone water and incubated in shaker at 28°C. Subsequently, 0.5ml of Nessler's reagent was added to the culture and the development of brown to yellow colour indicated a positive for ammonia production (Cappucino and Sherman, 1992).

Results and Discussion

Endophytic actinomycete isolates from medicinal plants

In the present study, from various tissue samples of six different medicinal plants, total 17 number of endophytic actinomycetes were obtained (Table 2). According to molecular 16S rRNA sequencing, the results showed that the isolates were classified into four families and four genera. Most of the isolates grouped into Streptomycetaceae (73.3%), followed by Nocardiopsaceae (13.3%), Tsukamurellaceae (6.6%) and Actinomycetaceae (6.6%) (Table 3).

In vitro antagonistic activity of the isolates

Among the 17 actinomycetes isolates, 8 (38%) (BPSEAC1, BPSEAC7, BPSEAC8, BPSEAC16, BPSEAC18, BPSEAC23, BPSEAC31 and BPSEAC33) isolates (Table 4) showed inhibitory activity against two important, i.e. *Fusarium oxysporum f.* sp. *ciceri* (MTCC-2791) and *Fusarium proliferatum* (MTCC-286) tested pathogens (Fig. 3).

Screening of plant growth promoting activities

Out of 17 isolates, 6 (35.2%) were able to solubilize inorganic phosphate and were considered as potential phosphate solubilizing isolates based on clear halo zone appearance around the colony on Pikovskaya's medium. The maximum halo zone was detected in the isolate BPSEAC4, BPSEAC5 and BPSEAC14 (Table 4). In this study, all strains showed positive for IAA production (Table 4). All 17 endophytic actinomycetes isolates were produced ammonia.

Isolation and identification of endophytic actinomycetes

To investigate the relationship among the more promising endophytic actinomycetes isolate, 16S rRNA gene sequences were aligned along with the sequences of type strains retrieved from DDBJ/ EMBL/ NCBI Gen Bank databases. Analysis of the 16S rRNA gene sequence by Blast N with 99-100% similarity confirmed that 10 isolates

could be members of genus Streptomyces (Table 3). Our results revealed that 16S rRNA amplification observed gene that Streptomyces formed a major group consistent with previous studies Zhao et al., 2011). Previous studies reported that endophytic microorganisms enter intact plant tissue by invagination of root hair cell wall, by penetration of the juncture between root hair and adjacent epidermal cells (Passari et al., 2015). The diversity of actinomycetes in the rhizosphere soils is positively correlated to the plant species (Germida et al., 1998; Hayakawa et al., 1988; Henis et al., 1986).Genus Streptomyces are frequently found actinomycetes in our study may be the present study medicinal plants rhizosphere form abundant and diverse microorganisms due to their high input of organic materials from plant roots. Genera like Nocardia, Tsukamurella and Actinobacteria species was isolated from medicinal plants were reported as rare endophytic actinomycetes (Qin et al., 2012). Similarly, in our study, sequences of the two isolates (BPSEAC3 and BPSEAC36) showed 99% identity to the sequences retrieved genus Nocardiopsis and the isolates (BPSEAC31 and BPSEAC43) showed high (99% identity each) to the genus Tsukamurella and Actinomycete, respectively. Genera Nocardiopsis, Tsukamurella Actinobacteria were among rare endophytic actinomycetes reported, offer novel source for bioactive compounds. Invitro antagonistic activity

In vitro antifungal activity of endophytic actinomycetes

All isolates were screened for their antagonistic activity against two major phytopathogenic fungi. Eight isolates (38%) showed antifungal activity against two tested pathogens. Similar, antigungal activity against fungi was reported by Verma *et al.*, 2009; Taechowisan and Lumyong, 2003).

Table.1 Summary of plant sample collection, taxonomic status, traditional medicinal value Rai and Lalramnghinglova, 2010, 2011

Scientific name	Local name in Mizo	Family	Habit	Traditional medicinal value
Senecioscandens BuchHam.	Saiekhlo	Asteraceae	Climber	Cancer/ ulcers
MikaniamicranthaKunth. WI	Japanhlo	Asteraceae	Climber	Haemostatic, fever, dysentery
Ageratum conizoides L.	Vailenhlo	Asteraceae	Herb	Stomach cancer, ant-diarrhoeal, aid in clotting of blood
Costusspeciosus (J. Konig) Sm.	Sumbul	Costaceae	Crepe-ginger	Kidney problem, leprosy, tonsillitis, kidney/gall baldder
Cassia fistula L.	Makpazangkang	Fabaceae	Tree	Pugatives, tonics, febrifuges
Scopariadulcis Medic	Hlothlum	Scrophulariaceae	Herb	Kidney stone, genitor-urinary troubles

Table.2 Morphological characteristics of endophytic actinomycetes and isolates obtained from different tissues of the medicinal plants

Isolates	Characteristics colony and cell morphology	Plant tis	Medium use	
BPSEAC1	Green in colour, powdery, hard -sticky, entire, irregular form, umbonate; colony with 1mm diameter.	Root	Mikania micrntha Kunth. WI	SCNA
BPSEAC3	White in colour, hard- sticky, entire, punctiform, convex; cplony with 0.5mm in diameter.	Stem	Mikania micrantha Kunth. WI	SCA
BPSEAC4	Cream colour, soft- sticky, circular form with a ring, umbonate and filamentous; colony with 1.8mm in diameter	Flower	Ageratum conyzoides L.	ISP ₅
BPSEAC5	Dark cream, soft, filamentous form with dark thick spot in the centre of the colony, flat and filiform; colony with 2mm in diameter	Rhizome	Costus speciosus(J. Konig) Sm	SCNA
BPSEAC7	Off-white, hard sticky, entire, irregular form, convex; colony with 1.2mm in diameter	Root	MikaniamicranthaKunth. WI	SCNA
BPSEAC8	Cream colour, hard, entire, spindle shape, raised; colony with 1mm in diameter	Root	MikaniamicranthaKunth. WI	ISP ₅
BPSEAC14	Dark-cream, hard -sticky, irregular form, flat, undulate; colony with 0.8mm in diameter	Stem	MikaniamicranthaKunth. WI	ISP ₅
BPSEAC16	Light orange, hard like a rubber, spindle form, pulvinate and curled; colony with 1mm in diameter	Root	Senecios candens BuchHam.	ISP ₅
BPSEAC18	Cream-white in colour, sticky, entire, flat shape and irregular form; colony with 1.1mm in diameter	Petiole	Cassia fistula L.	ISP ₅
BPSEAC21	Off-white, sticky, entire, concentric form, umbonate in elevation; colony with 1.2mm in diameter	Stem	Cassia fistula L.	ISP ₅
BPSEAC23	Pure white, soft -sticky, crateriform, undulate and filamentous; colony with 1mm in diameter	Root	Mikania micrantha Kunth. WI	SCA
BPSEAC31	Light orange, soft-sticky, punctiform, crateriform, entire; colony with 0.5mm in diameter	Petiole	Ageratum conyzoides L.	SCA
BPSEAC33	Cream-white powdery on top, hard-sticky, irregular form, umbonate, undulate; colony with 1mm in diameter	Root	Scopariadulcis Medic	TSA
BPSEAC36	with 0.8mm in diameter	Rhizome	Costus speciosus (J. Konig) Sm	SCA
BPSEAC37	Off-white, soft, flat, entire, circular shape; colony size with 2mm in diameter	Root	Scopariadulcis Medic	SCA
BPSEAC41	Creamy powdery, soft, irregular form with a ring, entire, flat; colony with 0.8mm in diameter	Root	Mikania micrantha Kunth. WI	SCNA
BPSEAC43	Cream, soft, filamentous form with ring, flat and irregular shape; colony with 0.8mm in diameter	Petiole	Senecios candensBuchHam.	SCA

Table 3 Phylogenetic relationship of endophytic actinomycetes

Isolates	Closest sequence	Similarity	Accession
Isolates	Closest sequence	Similarity	no.
BPSEAC1	Streptomyces somaliensis (KC98993)	99%	KU158241
BPSEAC3	Nocardiopis sp. (KM886195)	99%	KU158243
BPSEAC4	Streptomyces sp. (KP330251)	99%	KU158244
BPSEAC5	Streptomyces thermocarboxydus (KP128880)	99%	KU158245
BPSEAC7	Streptomyces sp. (JN408756)	99%	KU158247
BPSEAC8	Streptomyces sp. (KJ143641)	100%	KU158248
BPSEAC14	Streptomyces sp. (KM220610)	99%	KU158254
BPSEAC16	Streptomyces sp. (JQ422121)	96%	KU158256
BPSEAC18	Streptomyces sp. (JQ812094)	96%	KU158258
BPSEAC21	Streptomyces sp. (KP338793)	99%	KU158261
BPSEAC23	Streptomyces sp. (JQ812085)	95%	KU158263
BPSEAC31	Tsukamurella tyrosinosolvens (AB480761)	99%	KU158270
BPSEAC33	Streptomyces sp. (KR857308)	99%	KU158272
BPSEAC36	Nocardiopsis sp. (KF270095)	87%	KU158275
BPSEAC37	Streptomyces violascens (KT274752)	99%	KU158276
BPSEAC41	Streptomyces albidoflavus (KP339504)	99%	KU158280
BPSEAC43	Actinobacteria bacterium (KP053722)	99%	KU158282

Table.4 In vitro antagonism of the isolates along with their PGPR activities

Isolate	Antagonism against		Plant growth promoting activities (PGPR)		
	Fusarium oxysporumf.sp. ciceri (MTCC-2791)	Fusarium proliferatum (MTCC-286)	Phosphate solubilization	Indole-3-acetic acid	Ammonia
BPSEAC1	+	+		+	+
BPSEAC3				+	+
BPSEAC4				+	+
BPSEAC5			+++	+	+
BPSEAC7	+	+		+	+
BPSEAC8	+	+	++	+	+
BPSEAC14			+++	+	+
BPSEAC 16	+	+		+	+
BPSEAC18	+	+		+	+
BPSEAC21				+	+
BPSEAC23	+	+		+	+
BPSEAC31			++	+	+
BPSEAC33	+	+	+++	+	+
BPSEAC36				+	+
BPSEAC37			+	+	+
BPSEAC41				+	+
BPSEAC43				+	+

Fig.1a A representative endophytic actinomycetes from tissue of medicinal plants and **b** A representative pure culture endophytic actinomycetes of medicinal plants tissues



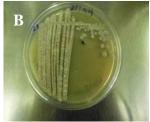


Fig.2 Field emission gun-scanning electron microscopy (FEG-SEM) micrographs of *Streptomyces* sp.

a BPSEAC4, b BPSEAC7, c BPSEAC8

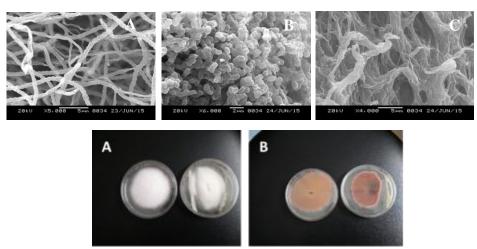


Fig. 3 Antagonistic activity of **a** BPSEAC23 against *Fusarium oxysporum f. sp. cice*ri (MTCC-2791) and **b** BPSEAC1 against *Fusarium proliferatum*(MTCC-286

All the antagonistic activity positive isolates were belonging to Streptomyces Metabolites of endophytic actinomycetes have extensive inhibition to the growth of fungal phytopathogens. Endophytic actinomycetes isolated from medicinal plants antifungal activities showed can investigated as a promising source of natural products as well as biocontrol agents. However, agricultural crops are prone destruction by various diseases caused by plant pathogens in the world. Actinomycetes with the ability to control plant pathogens may be the alternate sources of chemical fertilizers and pesticides in the agricultural programs.

Phosphate solubilisation of the isolates

Six isolates (35.2%),identified Streptomyces sp. (66.6%) and Tsukamurella detected (16.6%)was phosphate solubilisation activity. This may be either due to the endophytic actinomycetes secrete organic acids of low molecular weight which results in solubilisation of phosphate from insoluble complexes. Hence, endophytic actinomycetes producing phosphate solubilisations efficiency component in the development and plant growth. This result is consistent with findings of Oteino et al., (2015).

Indole-3-acetic acid (IAA) production

In this study, all the strains (100%) of the endophytic actinomycetes were showed positive for IAA production. Nimnoi and Pongslip (2009) reported that IAA synthetic bacteria enhanced root and shoot development of *Raphanus sativus* and *Brassica oleracea* more than fivefold when compared with the control. IAA producing actinomycetes may have important role in plant growth promotion.

Ammonia production

Here we detected all the isolates of actinomycetes endophytic produced ammonia. Marques et al., (2010) suggested that bacterial species producing ammonia plant growth. Bacteria enhances accumulate nitrogen and source to the host plant, enhanced elongation of roots and shoots of the host plant, consequently promoting plant biomass.

In conclusion, our results obtained diversity of endophytic actinomycetes. These results support the idea that medicinal plants in our study may signify favourable habitat for diversity of endophytic actinomycetes. Furthermore, this isolates producing the plant growth promoting activities and antifungal activity. Isolates ability to inhibit the major fungal phytopathogen may considered as a valuable candidate of biocontrol agent. These findings provide compelling evidence that the endophytic actinomycetes possess favourable source as efficient bioinoculents in the sustainable agricultural programs. Endophytic actinomycetes of medicinal plants are the key source of bioactive compounds. To the best of our knowledge first time reported that potential endophytic actinomycetes can be isolated from Senecios candens Buch.-Ham. and Mikania micrantha Kunth. WI. However. these plants may consider as a valuable actinomycetes. indicator of potential

Anticancer activity and antimicrobial activity with bacterial pathogens of these isolates will be examine in our further study.

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